Epidemiological Value of Isolating Bacteriophage in Outbreaks of Intestinal Infection*

ROY F. FEEMSTER, M.D., DR.P.H.
Massachusetts Department of Public Health, Boston, Mass.

THE bacteriophage has been largely a laboratory curiosity and a subject for controversy and speculation. The principal attempt to make use of it in a practical way has been as a curative agent, upon which opinions differ. Other useful applications of the phenomenon no doubt await discovery when more information is accumulated. With the purpose of determining whether there might be any public health applications of the phenomenon, a study was begun in 1932, undertaken largely because of the success with which the isolation of bacteriophage was applied in an outbreak of bacillary dysentery in a large institution (Medfield), the main features of which are reported in a paper now in press.[1]

In this epidemic it appeared that finding a bacteriophage in the stools from those affected which was active against the bacillus causing the disease was a useful means of aiding to differentiate these cases from those due to other causes. Stool cultures had proved unreliable for 2 reasons: (1) The bacillus was soon eliminated from the intestine, and when there was delay in obtaining specimens no organism was cultivated from them; (2) Specimens had to be mailed to Boston for examination, and even stools obtained at the proper time were often negative. The epidemic had begun early in the summer in one ward. A Hiss-Y dysentery bacillus was recovered from one patient but not from others. Isolation and general sanitary measures caused an abrupt cessation of cases.

Six weeks later the infection appeared again, this time in most of the buildings of the institution. During the second outbreak over 90 stools from clinical cases were examined, and from only 6 was the dysentery bacillus obtained. At the same time 81 stools were examined for bacteriophage and 29 were found positive. The figures are more striking when divided into groups according to the week of the disease. During the first 7 days bacilli were found in 28 per cent of the specimens examined, while bacteriophage was found in 33 per cent; during the second week bacteriophage was found in 80 per cent and bacilli in 13 per cent; during the third week bacteriophage was found in 45 per cent of specimens and bacilli in none. Thereafter neither was found with any regularity. Stools of individuals not affected did not contain bacteriophage. The bacteriophages isolated in this outbreak were active against all members of the Flexner group, as well as the Sonne and Shiga bacilli.

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Meanwhile an urgent necessity for finding other means of gaining information as to the etiology of outbreaks of gastroenteritis was felt. Such outbreaks are perhaps the most frequent variety that the epidemiologist is called upon to investigate, and in many instances, due to the short duration of illness, he does not arrive until the peak has passed and a majority of the cases are in the convalescent stage. Under such circumstances determniation of the etiological agent is difficult. Stool cultures are unsatisfactory because in most instances no suspicious organism is isolated. Even if one or two stools yield some organism which might be responsible, it is difficult to prove that the other cases have been caused by the same agent. Agglutination tests cannot be entirely depended upon, since some individuals may have agglutinins from previous attacks or sub-clinical exposures to the organism.

Since bacteriophage active against the invading organism has been demonstrated repeatedly in the stools of individuals suffering from illness due to various types of intestinal pathogens, it seemed that isolating bacteriophage from the stool might give some light as to the cause of an illness. Since the bacteriophage usually appears in the stool during the convalescent stage, the method would have the advantage of providing evidence long after the expectation of isolating the bacterium had passed. It was decided, therefore, to filter stools from these epidemics and set them up against a number of intestinal pathogens.

The usefulness of the procedure would depend upon several factors: (1) the chances of picking up a bacteriophage which had no relation to the outbreak; (2) the specificity of those isolated; (3) the regularity with which bacteriophages are recoverable from those ill, in contrast to those not affected.

In order to check up on the first factor, samples were collected from many sources, filtered, and placed in contact with a group of the more common intestinal bacteria. The bacteriophages isolated from these sources are summarized in Table I. It will be seen that except in sewage samples they are relatively rare. The chances of a single individual picking up a bacteriophage might be of some significance, but the chances of several individuals simultaneously picking up the same one would be quite small. That such is the case is indicated by the failure to find them in a large number of stools examined from persons involved in various outbreaks which have been studied not due to the dysentery bacillus.

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TABLE I

BACTERIOPHAGES ISOLATED FROM VARIOUS SOURCES IN MASSACHUSETTS

		Bacteriophages Isolated Active Against									
Source of Specimen	*Total Specimens Examined*	*B. typhosus*	*B. paratyphosus A*	*B. paratyphosus B*	*B. dysentery (Shiga)*	*B. dysentery (Flexner)*	*B. dysentery (Hiss-Y)*	*B. dysentery (Sonne)*	*B. enteritidis*	*B. coli communic*	*Staphylococcus*
Stools *	111	6	1	0	10	9	10	6	5	2	1
Sewage	89	34	11	11	52	42	51	39	20	30	4
Rivers	116	1	0	2	6	3	10	5	0	6	4
Lakes and ponds	70	0	0	0	4	2	0	1	0	3	3
Beaches (sea water)	44	0	0	1	5	0	1	2	0	0	0
Public water supplies	7	0	0	0	1	0	2	2	0	0	0
Total	437	41	12	14	78	56	74	55	25	41	12

* None of these stools were from dysentery outbreaks.

bacteriophages we find that they can be divided into several rough groups:

1. Those active against *B. typhosus* only
2. Those active against the Flexner dysentery group
3. Those active against all dysentery bacilli, including Shiga and Sonne
4. Those active against *B. typhosus, B. dysenteriae* (Shiga), *B. dysenteriae*(Sonne), and *B. enteritidis*
5. Those active against all of the group of intestinal pathogens used
6. Those active against *B. coli*

Not all can be placed in the above groups as there are considerable variations in valence. No doubt mixtures of bacteriophages were dealt with in some cases, particularly from sewage samples, but repeated platings of some of wide valence (Group 5) and isolations from plaques failed to narrow the valence. Perhaps more persistent efforts will reveal that some of them are mixtures.

Another fact which stood out conspicuously was that the colon bacillus was very seldom included in the valence of bacteriophages isolated with the intestinal pathogens. Frequently a colon bacteriophage was isolated from the same specimen that contained other phages, but it was usually quite specific for the colon bacillus, and the others did not often attack the colon bacillus. Most observers have remarked upon the regularity with which Shiga bacteriophages attack the colon bacillus, but this has not been true in the present study. The explanation is not yet apparent.

This irregular and wide valence adds uncertainty to the interpretation of isolations from stools until several are found and shown to be the same variety in different individuals in the same epidemic. In order to demonstrate that they are the same, it is necessary to determine their valence against a list of the commoner intestinal pathogens.

It has already been shown that bacteriophages are recoverable with great regularity from patients convalescing from bacillary dysentery and are absent from stools of those not affected. Whether this is true of other intestinal infections awaits demonstration.

Meanwhile outbreaks of gastroenteritis were occurring and attempts to make use of the method were being tried. Most of the outbreaks were mild to moderately severe diarrheas occurring in short, sharp epidemics, in contrast to the more drawn out course of bacillary dysentery in an institution. Those affected were not prostrated and few or none showed blood or mucus in the stool (see Table II). Stool cultures yielded an occasional organism not easily classified and also an occasional bacteriophage active against some of the pathogens used in the search. The same bacteriophage could not be recovered from a sufficient number of individuals to be significant, nor were those isolated ever active against the organisms found in the outbreaks. Either the intestinal disturbance was due to organisms not included in the list used, or the attacks were so mild that bacteriophages did not appear in the stool. We must not lose sight of the fact that some strains of bacilli are not susceptible to lysis by bacteriophage, though it does not seem plausible that all of the occasional organisms isolated from stools in these outbreaks could have happened to be insusceptible to lysis.

During one season semi-monthly samples were collected from 3 popular beaches near Boston during the spring before the bathing began, and weekly samples during the summer months. The plan was to examine these for bacteriophages to discover the usual ones present with the expectation that a new one might appear if an intestinal outbreak should occur among those frequenting the beaches. One hundred families at each beach were visited regularly and 273 physicians in the communities where the beaches were located agreed to report any intestinal disorders

TABLE II

OUTBREAKS OF INTESTINAL INFECTION

Outbreak	*Type*	*Population Involved*	*Cases*	*Causative Agent*	*Probable Mode of Spread*	*Character of Symptoms*	*Number of Specimens Examined*	*Isolation of Bacteriophage Useful*
Medfield	Institutional	2,100	100	*B. dys.* (Hiss-Y)	Contact and carriers	Violent diarrhea, fever mucus, blood	81	Yes
Williamsburg	Community	1,800	200	Not determined	Water	Purging, no mucus or blood	13	No
Fall River	Family	8	6	Not determined	Food	Purging, no mucus or blood	8	No
Westfield	Institutional	350	36	Not determined	Unknown	Vomiting, purging	45	No
Plymouth	Family	6	4	Not determined	Unknown	Vomiting, purging	6	No
Worcester	Family	7	5	*B. dys.* (Hiss-Y)	Food	Violent diarrhea, mucus and blood	6	Yes
Medfield	Institutional	2,100	500	Not determined	Food	Mild purging	19	No
Northfield	School	2,000	100	Not determined	Water	Mild purging	6	No
S. S. Columbian	Ship	50	40	Not determined	Food	Mild purging	15	No
Northampton	School	1,500	100	Not determined	Food	Mild purging	11	No
Fall River	Community	110,000	500	Not determined	Water	Mild purging	12	No
Danvers	Institutional	3,000	61	*B. dys.* (Hiss-Y)	Contact	Violent diarrhea, mucus and blood	65	Yes
Waverly	Institutional	2,000	156	*B. dys.*	Contact and food	Severe diarrhea, mucus and blood	47	Yes

which occurred. It happened that there was no outbreak among the groups studied during the season. Not even a single case of severe diarrhea was reported. The only information obtained, therefore, was the normal bacteriophage content of the waters of the beaches.

An outbreak in a family in Worcester, however, presented the same situation as in the institutional outbreak mentioned above. Three children were sent to a hospital with a very severe diarrhea. A dysentery bacillus was isolated from the most recent case. Bacteriophages were recovered from the stools of the other 2 children, and at a later date one was found in the stool of the child from whom the organism was isolated. Stools from 2 other children who had had mild attacks were examined and a bacteriophage was found in one. Here again isolation of bacteriophage aided in proving conclusively that all the children were suffering from the same illness.

During this summer outbreaks of bacillary dysentery occurred in 2 other institutions. In an outbreak in Danvers 13.4 per cent of the specimens examined for bacilli yielded a Hiss-Y dysentery bacillus and 55.2 per cent of the specimens examined for bacteriophage were positive. The percentage by weeks is shown in Table III. In an outbreak in Waverly bacilli were found in 10.7 per cent of the samples examined and bacteriophages in 49 per cent. This last outbreak occurred so late that more detailed figures are not yet available. The percentages given include examinations of samples from all cases of diarrhea occurring during the outbreaks, some of which were certainly not due to the dysentery bacillus. In isolating bacteriophages in these 2 outbreaks 2 markedly susceptible strains were used as well as a strain isolated from each institution. Several bacteriophages were obtained which would have been missed if the 2 stock strains had not been used. All of the bacteriophages were shown to be active against the organisms isolated from the institutions. Some of the dysentery bacilli isolated were found to be insusceptible to lysis by the bacteriophages at hand.

TABLE III

DYSENTERY BACILLI AND BACTERIOPHAGES ISOLATED FROM STOOLS
DANVERS OUTBREAK

	Dysentery Bacilli			*Bacteriophages*		
Week after onset	*Specimens examined*	*Number positive*	*Per cent positive*	*Specimens examined*	*Number positive*	*Per cent positive*
1	47	18	38.4	14	8	57.3
2	28	6	21.4	6	4	66.7
3	28	2	6.7	4	2	50.0
4	24	3	12.5	14	8	57.3
5	29	1	3.3	8	4	50.0
over 5	39	0	0	11	5	45.4
	195	30	13.4	58	32	55.2

DISCUSSION

It was a considerable source of disappointment that isolation of bacteriophages failed to be of aid in studying the more elusive epidemics of intestinal infection usually encountered. The fact that the method can be applied to bacillary dysentery, while interesting and of value in certain instances, is not so useful since the usual methods of studying such outbreaks are more satisfactory. However, it is not unlikely that by increasing the list of organisms used in testing, or by some other variation, the method may yet be made to yield information in such intestinal disturbances.

In making isolations from dysentery cases it is recommended that one or two

markedly susceptible strains of bacilli be used until it is ascertained that an organism recovered from a case in the outbreak will pick up bacteriophages from all stools in which they are present.

METHODS USED

Except for the time consuming operation of filtering through a Berkefeld filter, isolation of bacteriophages is no more complicated than isolating bacteria. Some of the ways of simplifying filtration were reported in a recent paper.[2]

COLLECTION OF SPECIMENS

The specimens should be collected without any differential or inhibiting fluid such as used in typhoid and other culture outfits. A satisfactory method is to saturate thoroughly and cover the cotton swabs in a diphtheria culture outfit. Drying does not appear to inactivate the bacteriophage at this stage.

ISOLATION INVOLVING FILTRATION

The simplest method of isolation is to place 5 to 10 c.c. of plain broth in contact with not more than 1 gm. of feces and after 15 to 30 minutes decant into a Berkefeld filter and pass through an "N" candle. To a tube of broth, seeded lightly with the organism causing the outbreak or with a stock strain, add 0.5 c.c. of the filtrate from the stool and incubate 18 to 24 hours. A control tube containing only the organism should be set up. If a strong bacteriophage is present, the tube containing the filtrate may be clear while the control will be turbid. If the bacteriophage is weak, no clearing may appear, in which case it is necessary to make sure no bacteriophage is present. There are 3 methods by which this may be done.

1. The most accurate method of checking up on doubtful tubes is to transfer 0.05 c.c. to an 18 hour broth culture of the organism, thoroughly mix, and then smear 0.1 c.c. evenly over the surface of an agar plate. This is best done with a bent glass rod sterilized by dipping in 95 per cent alcohol 2 or 3 times—each time allowing the alcohol to burn. The inoculating culture should be heavy enough to produce a complete film of growth, with no separate colonies visible. If bacteriophage is present, plaques will show in the bacterial film on the surface of the plate. Sometimes, however, a very active bacteriophage may completely inhibit growth, and there will be practically no colonies on the plate.

2. Another method is to smear a loopful of broth from the doubtful tube and another loopful from the control, each on separate spots about 1 cm. in diameter or larger on an agar plate. Lysis will be indicated by (1) no growth; (2) a scanty growth; or (3) plaques in the bacterial film. The amount of reduction in growth can be determined by comparing with the control.

3. The third way to check up on tubes showing no clearing is to seed thoroughly the surface of an agar plate with the organism. Then with a loopful of material from the tube make a circle on the surface of the agar. Make one also with the control for comparison. If bacteriophage is present, it will be indicated on the ring by more or less inhibition of growth of the organism or by a few well defined plaques. Usually the bacteriophage particles are much more numerous at 18 to 24 hours than the resistant organisms which have begun to grow out, and the latter do not interfere materially in interpreting the results on the plates.

ISOLATION WITHOUT FILTRATION

It is possible to isolate bacteriophages without filtering the material from the stool through a Berkefeld filter. The percentage of isolation will not be high, however, since only the most active ones

will be recovered in the presence of the rapid growing and spreading bacteria or of the spore forming organisms which will be encountered in stools. The stool specimen should be placed in contact with broth as outlined above, and 0.5 c.c. of the broth placed in an 18 hour broth culture of the organism causing the outbreak. After 12 to 24 hours incubation, the tube is placed in a water bath and the temperature held at 60° C. for ½ hour. This will usually kill the vegetative bacteria and not harm the bacteriophage. A very small loopful of the material is then placed in an 18 hour broth culture of the organism and immediately plated out as described above. The presence of bacteriophage will be shown by plaques or by complete inhibition of growth.

SPECEFICITY OF BACTERIOPHAGES

The bacteriophages isolated in this study were brought to full activity by serial transplanting in broth cultures of the susceptible organism and then filtering. The filtrates were placed in contact with the organisms named in Table I plus the 5 English strains of Flexner dysentery bacillus (V, W, X, Y, and Z). One drop of filtrate and 1 drop of broth culture of the organism is placed in a tube of broth. Controls receive only the organism.

A great deal of labor was saved by using special metal battery boxes, each holding two rows of 10 tubes each. These were modified from similar ones described by Wells,[3] the principal change being to leave the lower 2 inches of the tubes exposed instead of the middle, and to increase the number of tubes from 10 to 20 per box. These boxes eliminated handling cotton plugs when filling the tubes as well as when inoculating them. One row of tubes can be lifted up with a special lifter while observing the other. In using the boxes a separate row was used for each organism, 9 tubes receiving bacteriophages and the 10th being left as a control.

Another time-saving apparatus was devised to fill the tubes of broth more rapidly. It consists of a glass syringe and a special valve connected with a reservoir which automatically measures out the proper amount of broth when a tube is pushed against the bottom of the valve.

The tubes were left at room temperature for 18 to 24 hours. Incubation at 37° C. causes the resistant organisms to grow out more rapidly, and frequently tubes which have cleared within 6 to 12 hours will again become turbid by the end of 24 hours. At the end of 24 hours all tubes and controls were plated out on agar in numbered squares corresponding to the numbers of the tubes in the protocol. Sixteen to 20 squares can be marked off on a plate. An area about 1 cm. in diameter is evenly covered with a small loopful from each tube. Frequently bacteriophages are found on the plates which would have been missed because of failure of the broth tubes to become clear.

SUMMARY

In outbreaks of bacillary dysentery bacteriophages active against the organism causing the outbreak were isolated from the stools of 60 to 80 per cent of those showing clinical symptoms, when stools were obtained during the second week after onset.

In outbreaks due to other causes bacteriophage isolation did not yield any useful information.

BIBLIOGRAPHY

1. Feemster, Roy F. The Use of Bacteriophage in the Diagnosis of Bacillary Dysentery. *J. Infect. Dis.*, Oct., 1934.

2. Feemster, Roy F. Simplifying the Mechanics of Bacterial Filtration. *J. Lab. & Clin. Med.*, 19:786–789 (Apr.), 1934.

3. Wells, W. F. An Improved Fermentation Tube Battery. *A.J.P.H.*, 8:904–907, 1918.

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